Antioxidant Peptides

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BACKGROUND OF THE INVENTION

The present invention relates generally to a method for treating diseases associated with lipid oxidation. Specifically, the present invention is a new method for treating atherosclerosis and other oxidative disorders.

It is generally recognized that high blood cholesterol levels are significant risk factors in cardiovascular disease. Coronary heart disease (CHD) remains the leading cause of death in the industrialized countries. Despite recent declines in CHD mortality, CHD is still responsible for more than 500,000 deaths in the U.S. annually. It is estimated that CHD, directly and indirectly, costs the U.S. more than $100 billion a year. The primary cause of CHD is atherosclerosis, a disease characterized by the deposition of lipids in the arterial vessel wall, resulting in a narrowing of the vessel passages and ultimately hardening the vascular system.

Atherosclerosis as manifested in its major clinical complication, ischaemic heart disease, is thought to begin with local injury to the arterial endothelium followed by proliferation of arterial smooth muscle cells from the medial layer to the intimal layer

along with deposition of lipid and accumulation of foam cells in the lesion. As the atherosclerotic plaque develops, it progressively occludes more and more blood vessel and can eventually lead to ischaemia or infarction. Therefore, it is desirable to provide a method of inhibiting the progression of atherosclerosis in patients in need thereof.

The atherosclerosis is a process of degeneration of the arterial wall and represents the main cause of many vascular pathologies like ischaemic cardiopathies (angina pectoris and myocardial infarction) and cerebral thrombosis, main causes of death in the industrial countries.

Many efforts have been carried out to understand the etiology of this pathology of wide diffusion and importance and to find out the possible therapeutic treatments.

The atherosclerosis is a process with composite etiology that involves in varied measure different factors and cell types. Ross R. in New Engl. J. Med., 314, 488-495, (1986) assumes that the endothelial damages, caused by exposure to many risk factors, represent the main event in the genesis of the atheroma and in Amer. J. Pathol., 143, 987-1001, (1993) finds out an important cause of the induction of such endothelial damage and of the progress and complication of the atheromatous plaque in the high serum rates of lipids and cholesterol that characterize the hyperlipidaemias and that often aggravate the diabetic pathologies. The pharmacological interventions aimed at lowering the plasma levels of cholesterol and low density lipoproteins (LDL) have proved to be effective in the prevention of the vascular coronary pathologies and in the treatment of atheromatous plaques, as reported by Steinberg D. in J.A.M.A., 253, 2080-2086, (1985) and in Atherosclerosis, 3, 283-301, (1983). The atherogenic risk joined to the LDL appears bound together with not only their plasma concentration but also their qualitative

characteristics; the possible modifications of the structure and composition of the LDL in the plasma or in the arterial wall can in fact make these macromolecules more atherogenic, namely more apt to trigger off and to stimulate the formation of the atheromatous plaque, as reported by Steinberg D. in New Engl. J. Med., 320, 915-924, (1989). The oxidative processes that occur by action of oxidizing agents present in the plasma or in the endothelial cells of the arterial wall are of main importance among the modifications that the LDL can undergo in vivo. Many in vitro and in vivo experiments, together with the results of epidemiological investigations, support the hypothesis that this mechanism represent a key event in the development of the atherosclerosis, as reported by Esterbauer H. *et al.* in Brit. Med. Bull., 49, 566-576, (1993).

The endogenous antioxidizers challenge the chain of propagation by means of an effective scavenging of the peroxylic radicals and the concentration of hydroperoxides increases only when the endogenous antioxidizers are depleted (latency period).

The reactive species of oxygen that form during the process of oxidation together with monounsaturated aldehydes that stem from the decomposition of the hydroperoxides, mainly malondialdehyde (MDA) and 4-hydroxynonenal, cause important changes in the primary structure of the main LDL apoprotein, the B-100 apolipoprotein (apo B-100). Such modification, by helping the LDL absorption by the macrophages, causes the intracellular accumulation of esters of the cholesterol and the formation of foam cells with subsequent development of the atherosclerotic plaque, as reported by Vanderyse L., *et al.* in Atherosclerosis, 97, 187-199, (1992).

Steinberg D. *et al.* in Proc. Nat. Acad. Sci. USA, 84, 7725-7729, (1987) show how compounds with antioxidizing action towards the LDL can have a part as anti-

atherosclerotic drugs. The effectiveness of a treatment by means of substances having antioxidizing action is based on observations limited to in vitro and animal models and on the in vivo use of the Vitamin E. In fact the atherogenic potentialities of the LDL are remarkably lowered in vitro when the LDL are incubated in presence of antioxidizers, like for instance probucol or Vitamin E, as reported by Esterbaur H. *et al.* in Amer. J. Clin. Nutr., 53, 314S-321S, (1991).

Therefore, on the basis of the above mentioned considerations, it is reasonable to suppose that molecules having both hypolipidemic and antioxidant activity represent a therapeutic development important in the treatment of the disorders of the lipoproteic metabolism and in the prevention of the atherosclerosis.

The compounds object of the present invention are endowed with antioxidant and hypolipidaemic properties, in particular they show the capability to prevent and/or to delay the oxidative modification of the LDL, *i.e.* they compete with the chain of propagation of the lipidic peroxidation through an effective scavenging of the peroxylic radicals.

The oxidative modifications of the low density lipoproteins (LDL) represent, as it has already been seen, a key event in the pathogenesis of the atherosclerosis and therefore the compounds object of the present invention can find useful therapeutical application in the treatment of the atherosclerosis and in the prevention of many vascular pathologies like ischaemic cardiopathies (angina pectoris and myocardial infarction), cerebral thrombosis and peripheral arteriopathies.

While the causative factors in the development of atherosclerosis are many, two important ones are elevated serum cholesterol levels and excessive LDL oxidation.

SUMMARY OF THE INVENTION

The present invention provides a method and means for inhibiting lipid oxidation.

In one embodiment, pharmacological composition will comprise an apolipoprotein (apo) A-IV compound, derivative or fragment thereof with a pharmaceutically acceptable carrier, fillers or excipients. The administering step may comprise administering a pharmacological composition comprising an apolipoprotein (apo) A-IV compound, derivative or fragment thereof along with pharmaceutically acceptable carrier, fillers or excipients.

The method may be by oral administration of an apolipoprotein (apo) A-IV compound, derivative or fragment thereof or a pharmaceutically acceptable salt or derivative thereof into said mammal.

The administering step comprises parenteral administration of the an apolipoprotein (apo) A-IV compound, derivative or fragment thereof or a pharmaceutically acceptable salt or derivative thereof into said mammal. This administration may be by transdermal administration, subcutaneous injection, intravenous injection, intraperitoneal injection, intramuscular injection, intrasternal injection, intrathecal injection, intraventricular and intracerebroventricular injection and infusion techniques.

The methods also comprise administering an apolipoprotein (apo) A-IV compound, derivative or fragment thereof or a pharmaceutically acceptable salt or derivative thereof along with a solvent or carrier. The lipophilic solvent or carrier may be an organic solvent, phosphatidyl choline and cholesterol.

Accordingly, an object of the present invention is to provide treating a mammal having a condition associated with lipid oxidation.

In addition, a number of novel lipid oxidation suppressant peptides, derived from apolipoprotein A-IV, have been made. These peptides possess lipid oxidation inhibiting properties which when administered orally or intravenously, can be used to decrease atherosclerosis. Since the peptides comprise specific portions of the native apo A-IV protein, there should be no immunogenicity problems associated with their administration to humans.

The peptides of the present invention correspond to specific areas of the apolipoprotein A-IV molecule and comprise at least a six amino acid sequence derived from the amino terminal portion of the mature apolipoprotein A-IV. Larger peptides of 15 and 90 amino acids, each containing within its sequence the aforementioned repeat sequence are also contemplated by the present invention.

By "homologues" is meant the corresponding peptides derived from other known apo A-IV proteins and having the same or substantially the same lipid oxidation inhibition properties. By "analogs" is meant substitutions in the amino acid sequences of the peptides, providing the lipid oxidation inhibition properties are retained. Analogs may also encompass additional amino acids, added to the N- and/or C-terminal portion of the peptide. For example, analogs of the peptides of the invention may contain cysteine or another amino acid, at the amino or carboxyl end of the peptide by which the peptide may be covalently attached to a carrier protein, *e.g.*, albumin, for in vivo administration. Other carrier molecules include polyethylene glycol (PEG) which functions to avoid proteolytic cleavage and clearing of peptides from the blood.

The peptides of the present invention may be linked to an additional sequence of amino acids by either or both the N-terminus and the C-terminus, wherein the additional sequences are from 1 to about 45 amino acids in length. Such additional amino acid sequences, or linker sequences can be conveniently affixed to a detectable label or solid matrix, or carrier. Labels, solid matrices and carriers that can be used with peptides of the present invention are described below. Typical amino acid residues used for linking are tyrosine, cysteine, lysine, glutamic acid and aspartic acid, or the like.

In another embodiment of the invention, the lipid oxidation inhibiting peptides of the present invention substantially correspond to the following amino acid sequences:

(A) MFLKAVVLTV ALVAITGTQA EVTSDQVANV;

(B) MWDYFTQLSN NAKEAVEQLQ KTDVTQQLNT LFQDKLGNIN TYADDLQNKL VPFAVQLSGH LTKETERVRE EIQKELEDLR AMVI;

(C) MLPHANKVSQ;

(D) MFGDNVQKLQ EHLRPYATDL QAQINAQTQD;

(E) MKRQLTPYIQR; SEQ ID 5

(F) MQTTIQDNVE NLQSS;

(G) MVPFANELKE KFNQN;

(H) MEGLKGQLTP
RANELKATID
QNLEDLRSRL
APLAEGVQEK
LNIHQ;

(I) MEGLAFQ;

(J) MKKNAEELHT
KVSTNIDQLQ
KNLAPLVEDV
QSKLKGNTEG
LQKSLEDLNK
QLDQQVEVFR
RAVEPLGDKFN;

(K) MALVQQ;

(L) MEKFRQQLGS
DSGDVESHLS
FLEKNLREKV
SSF;

(M) MSTLQKKGSP
DQPLALPLPE
QVQEQVQEQV
QPKPLES;

as well as homologues and analogs thereof; wherein:

A =	Ala	= Alanine
R =	Arg	= Arginine
N =	Asn	= Asparagine
D =	Asp	= Aspartic acid
B =	Asx	= Asparagine or aspartic acid
C =	Cys	= Cysteine
Q =	Gin	= Glutamine
E =	Glu	Glutamic acid
Z =	Glx	= Glutamine or Glutamic acid
G	Gly	= Glycine
H =	His	= Histidine
I =	Ile	= Isoleucine
L =	Leu	= Leucine

K =	Lys	= Lysine
F =	Phe	= Phenylalanine
P =	Pro	= Proline
S =	Ser	= Serine
T =	Thr	= Threonine
W =	Trp	= Tryptophan
Y =	Tyr	= Tyrosine
V =	Val	= Valine

The sequences of the peptides listed above under (A), (B), (C), (D), (E), (F), (G), (H), (I), (J), (K), (L), and (M), are set forth as SEQ ID NO: 1-13 respectively.

The one-letter symbols used to represent the amino acid residues in the peptides of the present invention are those symbols commonly used in the art. By "substantially corresponding" is meant an amino acid sequence having a homology to any of the listed sequences of about 70%.

The present invention also provides compositions for lipid oxidation inhibition in animals, including man. The compositions have as their active ingredients, at least one of the above peptides according to the present invention, admixed with a physiologically acceptable carrier. The term "pharmaceutically acceptable" refers to a molecular entity or composition that does not produce an allergic or similar unwanted reaction when administered to animals or humans.

The pharmaceutically acceptable carriers used in conjunction with the peptides of the present invention vary according to the mode of administration. For example, the compositions may be formulated in any suitable carrier for oral liquid formulation such as suspensions, elixirs and solutions. Compositions for liquid oral dosage include any of the usual pharmaceutical media such as, for example, water, oils, alcohols, flavoring agents, preservatives, coloring agents and the like. In the case of oral solid preparations (powder

capsules and tablets) carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like may be used. In addition, carriers such as liposomes, microemulsions and self emulsifiable glasses may be used.

The compositions of the present invention may also be formulated for intravenous administration. In this instance, the peptides are admixed with sterile water and saline or other pharmaceutically acceptable carrier.

The peptides of the present invention may be altered with modifying structures such as PEG to prevent both proteolysis of the peptides and reduce the clearing of peptides from the blood.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: **Inhibition effect of different doses of apoA-IV on copper mediated oxidation of fasting lymph and LDL.** Fasting intestinal lymph or LDL were oxidized in the absence or presence of apoA-IV at the concentration indicated. Fasting lymph was oxidized by 100 μM $CuSO_4$ for 3 h at 37°C( ). LDL (50 μg/ml) was oxidized by 10 μM $CuSO_4$ for 3 h at 37°C(○). Lipid peroxidation was measured by the formation of TBARS

and expressed as the percentage of protection. Results are means ± S.E. of three measurements. * $p<0.05$ and ** $p<0.01$ compared to control.

Fig. 2: **Kinetics of LDL oxidation in the presence or absence of apolipoproteins.** LDL (50 µg/ml) was oxidized using 10 µM $CuSO_4$ at 30°C in a quartz cuvette. Absorbance readings at 234 nm were taken every 5 min to measure the formation of conjugated dienes. Conjugated diene formation in LDL was determined in the absence of apolipoproteins (❑), or in the presence of 2.5 µg/ml of apoA-IV (○), 5 µg/ml of apoA-IV (●), rat apoE (Δ) or human apoE (▲).

Fig. 3: **Inhibitory effect of apoA-IV during the propagation phase of LDL oxidation.** LDL oxidation, monitored by the conjugated diene assay, was allowed to proceed to the propagation phase of oxidation. ApoA-IV was added to the oxidation reaction during the propagation phase, and conjugated dienes were continuously measured in samples containing only LDL (□), LDL plus tris-saline (×), LDL plus 5 µg/ml apoA-IV (Δ), LDL plus 10 µg/ml apoA-IV (○), and LDL plus 20 µg/ml apoA-IV (●). The inset shows the change in slope before and after the addition of apoA-IV.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for a number of lipid oxidation inhibiting peptides of approximately 5-90 amino acids in length, which substantially correspond in sequence to amino acid sequence found in specific portions of apolipoprotein A-IV ("apo A-IV").

Atherosclerosis is a disease state characterized by the development and growth of atherosclerotic lesions or plaque. The identification of those patients who are in need of treatment for atherosclerosis is well within the ability and knowledge of one of ordinary skill in the art. For example, individuals who are either suffering from clinically significant atherosclerosis or who are at risk of developing clinically significant atherosclerosis are patients in need of treatment for atherosclerosis. A clinician of ordinary skill in the art can readily determine, by the use of clinical tests, physical examination and medical/family history, if an individual is a patient in need of treatment for atherosclerosis.

An effective anti-atherosclerotic amount is an amount which is effective in inhibiting the development or growth of atherosclerosis in a patient in need thereof. As such, successful treatment of a patient for atherosclerosis is understood to include effectively slowing, interrupting, arresting, or stopping atherosclerotic lesion or plaque development or growth and does not necessarily indicate a total elimination of atherosclerosis. It is further understood and appreciated by those of ordinary skill in the art that successful treatment for atherosclerosis can include prophylaxis in preventing atherosclerotic lesion or plaque formation.

Peroxidation of LDL lipid, such as the unsaturated fatty acid portions of LDL cholesteryl esters and phospholipids, is known to facilitate the deposition of cholesterol in macrophages which subsequently are deposited in the vessel wall and are transformed into

foam cells. The identification of those patients who are in need of inhibition of peroxidation of LDL lipid is well within the ability and knowledge of one of ordinary skill in the art. For example, those individuals who are in need of treatment for atherosclerosis as defined hereinabove, are also patients who are in need of inhibition of peroxidation of LDL lipid. An effective antioxidant amount is an amount which is effective in inhibiting the peroxidation of LDL lipid in a patient's blood.

As used herein, the term "patient" refers to a warm-blooded animal or mammal which is in need of treatment for a chronic heart disease, atherosclerosis, hypercholesterolemia or which is in need of inhibiting oxidation.

A "therapeutically effective amount" is an amount which is effective, upon single or multiple dose administration to the patient, in providing relief of symptoms associated with atherosclerotic diseases.

As used herein, "relief of symptoms" refers to decrease in severity over that expected in the absence of treatment and does not necessarily indicate a total elimination or cure of the disease. Relief of symptoms is also intended to include prophylaxis.

As used herein, "peptide" refers to a linear series of less than about 100 amino acid residues connected to one another by peptide bonds between the alpha-amino and carboxy groups of adjacent amino acid residues. The term "synthetic peptide" is intended to refer to a chemically derived chain of amino acid residues linked together by peptide bonds and which is free of naturally occurring proteins and fragments thereof. Additionally, analogs, homologues, fragments, chemical derivatives and pharmaceutically acceptable salts of the novel peptides provided herein are included within the scope of the term "peptide".

By "peptide analog" is meant a peptide which differs in amino acid sequence from the native peptide only by conservative amino acid substitutions, for example, substitution of Leu for Val, or Arg for Lys, *etc.*, or by one or more non-conservative amino acid substitutions, deletions, or insertions located at positions which do not destroy the biological activity of the peptide (in this case, the ability of the peptide to target vascular lesions). A peptide analog, as used herein, may also include, as part or all of its sequence, one or more amino acid analogues, molecules which mimic the structure of amino acids, and/or natural amino acids found in molecules other than peptide or peptide analogues.

By "homologues" is meant the corresponding peptides derived from other known apo A-IV proteins having the same or substantially the same appetite suppressant and feeding inhibition properties.

By "analogs" is meant substitutions or alterations in the amino acid sequences of the peptides of the invention, which substitutions or alterations do not abolish the appetite suppressant or feeding inhibition properties of the peptides. Thus, an analog might comprise a peptide having a substantially identical amino acid sequence to a peptide provided herein as SEQ ID NO:1-13 and in which one or more amino acid residues have been conservatively substituted with chemically similar amino acids. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another. Likewise, the present invention contemplates the substitution of one polar (hydrophilic) residue such as between arginine and lysine, between glutamine and asparagine, and between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for

another or the substitution of one acidic residue such as aspartic acid or glutamic acid for another is also contemplated.

The phrase "conservative substitution" also includes the used of chemically derivatized residues in place of a non-derivatized residues as long as the peptide retains the requisite appetite suppressant or feeding inhibition properties. Analogs also include the presence of additional amino acids or the deletion of one or more amino acids which do not affect biological activity. For example, analogs of the subject peptides may contain an N- or C-terminal cysteine, by which, if desired, the peptide may be covalently attached to a carrier protein, *e.g.*, albumin. Such attachment, it is believed, will minimize clearing of the peptide from the blood and also prevent proteolysis of the peptides.)

The practice of the present invention employs, unless otherwise indicated, conventional techniques of synthetic organic chemistry, protein chemistry, molecular biology, microbiology, and recombinant DNA technology, which are well within the skill of the art. Such techniques are explained fully in the literature.

In preferred embodiments, the peptide or peptide analog is water soluble; or is soluble in a physiological fluid, preferably, one which is at physiological pH, for example, blood plasma.

In another preferred embodiment, the peptide has a molecular conformation analogous to the molecular conformation (size, shape, charge) of a surface region of the apolipoprotein A-IV (apo A-IV) moiety.

Examples of preferred peptide or peptide analogues include:

Met-Phe-Leu-Lys-Ala-Val-Val-Leu-Thr-Val-Ala-Leu-Val-Ala-Ile-Thr-Gly-Thr-Gln-Ala-Glu-Val-Thr-Ser-Asp-Gln-Val-Ala-Asn-Val (SEQ ID NO:1);

Met-Trp-Asp-Tyr-Phe-Thr-Gln-Leu-Ser-Asn-Asn-Ala-Lys-Glu-Ala-Val-Glu-Gln-Leu-Gln-Lys-Thr-Asp-Val-Thr-Gln-Gln-Leu-Asn-Thr-Leu-Phe-Gln-Asp-Lys-Leu-Gly-Asn-Ile-Asn-Thr-Tyr-Ala-Asp-Asp-Leu-Gln-Asn-Lys-Leu-Val-Pro-Phe-Ala-Val-Gln-Leu-Ser-Gly-His-Leu-Thr-Lys-Glu-Thr-Glu-Arg-Val-Arg-Glu-Glu-Ile-Gln-Lys-Glu-Leu-Glu-Asp-Leu-Arg-Ala-Met-Val-Ile (SEQ ID NO:2);

Met-Leu-Pro-His-Ala-Asn-Lys-Val-Ser-Gln (SEQ ID NO:3);

Met-Phe-Gly-Asp-Asn-Val-Gln-Lys-Leu-Gln-Glu-His-Leu-Arg-Pro-Tyr-Ala-Thr-Asp-Leu-Gln-Ala-Gln-Ile-Asn-Ala-Gln-Thr-Gln-Asp (SEQ ID NO:4);

Met-Lys-Arg-Gln-Leu-Thr-Pro-Tyr-Ile-Gln-Arg (SEQ ID NO:5);

Met-Gln-Thr-Thr-Ile-Gln-Asp-Asn-Val-Glu-Asn-Leu-Gln-Ser-Ser (SEQ ID NO:6);

Met-Val-Pro-Phe-Ala-Asn-Glu-Leu-Lys-Glu-Lys-Phe-Asn-Gln-Asn (SEQ ID NO:7);

Met-Glu-Gly-Leu-Lys-Gly-Gln-Leu-Thr-Pro-Arg-Ala-Asn-Glu-Leu-Lys-Ala-Thr-Ile-Asp-Gln-Asn-Leu-Glu-Asp-Leu-Arg-Ser-Arg-Leu-Ala-Pro-Leu-Ala-Glu-Gly-Val-Gln-Glu-Lys-Leu-Asn-Ile-His-Gln (SEQ ID NO:8);

Met-Glu-Gly-Leu-Ala-Phe-Gln (SEQ ID NO:9);

Met-Lys-Lys-Asn-Ala-Glu-Glu-Leu-His-Thr-Lys-Val-Ser-Thr-Asn-Ile-Asp-Gln-Leu-Gln-Lys-Asn-Leu-Ala-Pro-Leu-Val-Glu-Asp-Val-Gln-Ser-Lys-Leu-Lys-Gly-Asn-Thr-Glu-Gly-Leu-Gln-Lys-Ser-Leu-Glu-Asp-Leu-Asn-Lys-Gln-Leu-Asp-Gln-Gln-Val-Glu-Val-Phe-Arg-Arg-Ala-Val-Glu-Pro-Leu-Gly-Asp-Lys-Phe-Asn (SEQ ID NO:10);

Met-Ala-Leu-Val-Gln-Gln (SEQ ID NO:11);

Met-Glu-Lys-Phe-Arg-Gln-Gln-Leu-Gly-Ser-Asp-Ser-Gly-Asp-Val-Glu-Ser-His-Leu-Ser-Phe-Leu-Glu-Lys-Asn-Leu-Arg-Glu-Lys-Val-Ser-Ser-Phe (SEQ ID NO:12);

Met-Ser-Thr-Leu-Gln-Lys-Lys-Gly-Ser-Pro-Asp-Gln-Pro-Leu-Ala-Leu-Pro-Leu-Pro-Glu-Gln-Val-Gln-Glu-Gln-Val-Gln-Glu-Gln-Val-Gln-Pro-Lys-Pro-Leu-Glu-Ser (SEQ ID NO:13);

and derivatives, analogues, homologues, fragments and mixtures thereof.

In various other preferred embodiments, the peptide or peptide analog has an acetylated amino terminus and/or an amidated carboxy terminus. Examples of such peptide or peptide analogues include:

H2N-Met-Phe-Leu-Lys-Ala-Val-Val-Leu-Thr-Val-Ala-Leu-Val-Ala-Ile-Thr-Gly-Thr-Gln-Ala-Glu-Val-Thr-Ser-Asp-Gln-Val-Ala-Asn-Val-CONH2 (SEQ ID NO:1);

CH3CONH-Met-Phe-Leu-Lys-Ala-Val-Val-Leu-Thr-Val-Ala-Leu-Val-Ala-Ile-Thr-Gly-Thr-Gln-Ala-Glu-Val-Thr-Ser-Asp-Gln-Val-Ala-Asn-Val-CONH2 (SEQ ID NO:1);

H2N-Met-Trp-Asp-Tyr-Phe-Thr-Gln-Leu-Ser-Asn-Asn-Ala-Lys-Glu-Ala-Val-Glu-Gln-Leu-Gln-Lys-Thr-Asp-Val-Thr-Gln-Gln-Leu-Asn-Thr-Leu-Phe-Gln-Asp-Lys-Leu-Gly-Asn-Ile-Asn-Thr-Tyr-Ala-Asp-Asp-Leu-Gln-Asn-Lys-Leu-Val-Pro-Phe-Ala-Val-Gln-Leu-Ser-Gly-His-Leu-Thr-Lys-Glu-Thr-Glu-Arg-Val-Arg-Glu-Glu-Ile-Gln-Lys-Glu-Leu-Glu-Asp-Leu-Arg-Ala-Met-Val-Ile-CONH2 (SEQ ID NO:2);

CH3CONH-Met-Trp-Asp-Tyr-Phe-Thr-Gln-Leu-Ser-Asn-Asn-Ala-Lys-Glu-Ala-Val-Glu-Gln-Leu-Gln-Lys-Thr-Asp-Val-Thr-Gln-Gln-Leu-Asn-Thr-Leu-Phe-Gln-Asp-Lys-Leu-Gly-Asn-Ile-Asn-Thr-Tyr-Ala-Asp-Asp-Leu-Gln-Asn-Lys-Leu-Val-Pro-Phe-Ala-Val-Gln-Leu-Ser-Gly-His-Leu-Thr-Lys-Glu-Thr-Glu-Arg-Val-Arg-Glu-Glu-Ile-Gln-Lys-Glu-Leu-Glu-Asp-Leu-Arg-Ala-Met-Val-Ile-CONH2 (SEQ ID NO:2);

H2N-Met-Leu-Pro-His-Ala-Asn-Lys-Val-Ser-Gln-CONH2 (SEQ ID NO:3);

CH3CONH-Met-Leu-Pro-His-Ala-Asn-Lys-Val-Ser-Gln-CONH2 (SEQ ID NO:3);

H2N-Met-Phe-Gly-Asp-Asn-Val-Gln-Lys-Leu-Gln-Glu-His-Leu-Arg-Pro-Tyr-Ala-Thr-Asp-Leu-Gln-Ala-Gln-Ile-Asn-Ala-Gln-Thr-Gln-Asp-CONH2 (SEQ ID NO:4);

CH3CONH-Met-Phe-Gly-Asp-Asn-Val-Gln-Lys-Leu-Gln-Glu-His-Leu-Arg-Pro-Tyr-Ala-Thr-Asp-Leu-Gln-Ala-Gln-Ile-Asn-Ala-Gln-Thr-Gln-Asp-CONH2 (SEQ ID NO:4);

H2N-Met-Lys-Arg-Gln-Leu-Thr-Pro-Tyr-Ile-Gln-Arg-CONH2 (SEQ ID NO:5);

CH3CONH-Met-Lys-Arg-Gln-Leu-Thr-Pro-Tyr-Ile-Gln-Arg-CONH2 (SEQ ID NO:5);

H2N-Met-Gln-Thr-Thr-Ile-Gln-Asp-Asn-Val-Glu-Asn-Leu-Gln-Ser-Ser-CONH2 (SEQ ID NO:6);

CH3CONH-Met-Gln-Thr-Thr-Ile-Gln-Asp-Asn-Val-Glu-Asn-Leu-Gln-Ser-Ser-CONH2 (SEQ ID NO:6);

H2N-Met-Val-Pro-Phe-Ala-Asn-Glu-Leu-Lys-Glu-Lys-Phe-Asn-Gln-Asn-CONH2 (SEQ ID NO:7);

CH3CONH-Met-Val-Pro-Phe-Ala-Asn-Glu-Leu-Lys-Glu-Lys-Phe-Asn-Gln-Asn-CONH2 (SEQ ID NO:7);

H2N-Met-Glu-Gly-Leu-Lys-Gly-Gln-Leu-Thr-Pro-Arg-Ala-Asn-Glu-Leu-Lys-Ala-Thr-Ile-Asp-Gln-Asn-Leu-Glu-Asp-Leu-Arg-Ser-Arg-Leu-Ala-Pro-Leu-Ala-Glu-Gly-Val-Gln-Glu-Lys-Leu-Asn-Ile-His-Gln-CONH2 (SEQ ID NO:8);

CH3CONH-Met-Glu-Gly-Leu-Lys-Gly-Gln-Leu-Thr-Pro-Arg-Ala-Asn-Glu-Leu-Lys-Ala-Thr-Ile-Asp-Gln-Asn-Leu-Glu-Asp-Leu-Arg-Ser-Arg-Leu-Ala-Pro-Leu-Ala-Glu-Gly-Val-Gln-Glu-Lys-Leu-Asn-Ile-His-Gln-CONH2 (SEQ ID NO:8);

H2N-Met-Glu-Gly-Leu-Ala-Phe-Gln-CONH2 (SEQ ID NO:9);

CH3CONH-Met-Glu-Gly-Leu-Ala-Phe-Gln-CONH2 (SEQ ID NO:9);

H2N-Met-Lys-Lys-Asn-Ala-Glu-Glu-Leu-His-Thr-Lys-Val-Ser-Thr-Asn-Ile-Asp-Gln-Leu-Gln-Lys-Asn-Leu-Ala-Pro-Leu-Val-Glu-Asp-Val-Gln-Ser-Lys-Leu-Lys-Gly-Asn-Thr-Glu-Gly-Leu-Gln-Lys-Ser-Leu-Glu-Asp-Leu-Asn-Lys-Gln-Leu-Asp-Gln-Gln-Val-Glu-Val-Phe-Arg-Arg-Ala-Val-Glu-Pro-Leu-Gly-Asp-Lys-Phe-Asn-CONH2 (SEQ ID NO:10);

CH3CONH-Met-Lys-Lys-Asn-Ala-Glu-Glu-Leu-His-Thr-Lys-Val-Ser-Thr-Asn-Ile-Asp-Gln-Leu-Gln-Lys-Asn-Leu-Ala-Pro-Leu-Val-Glu-Asp-Val-Gln-Ser-Lys-Leu-Lys-Gly-Asn-Thr-Glu-Gly-Leu-Gln-Lys-Ser-Leu-Glu-Asp-Leu-Asn-Lys-Gln-Leu-Asp-Gln-Gln-Val-Glu-Val-Phe-Arg-Arg-Ala-Val-Glu-Pro-Leu-Gly-Asp-Lys-Phe-Asn-CONH2 (SEQ ID NO:10);

H2N-Met-Ala-Leu-Val-Gln-Gln-CONH2 (SEQ ID NO:11);

CH3CONH-Met-Ala-Leu-Val-Gln-Gln-CONH2 (SEQ ID NO:11);

H2N-Met-Glu-Lys-Phe-Arg-Gln-Gln-Leu-Gly-Ser-Asp-Ser-Gly-Asp-Val-Glu-Ser-His-Leu-Ser-Phe-Leu-Glu-Lys-Asn-Leu-Arg-Glu-Lys-Val-Ser-Ser-Phe-CONH2 (SEQ ID NO:12);

CH3CONH-Met-Glu-Lys-Phe-Arg-Gln-Gln-Leu-Gly-Ser-Asp-Ser-Gly-Asp-Val-Glu-Ser-His-Leu-Ser-Phe-Leu-Glu-Lys-Asn-Leu-Arg-Glu-Lys-Val-Ser-Ser-Phe-CONH2 (SEQ ID NO:12);

H2N-Met-Ser-Thr-Leu-Gln-Lys-Lys-Gly-Ser-Pro-Asp-Gln-Pro-Leu-Ala-Leu-Pro-Leu-Pro-Glu-Gln-Val-Gln-Glu-Gln-Val-Gln-Glu-Gln-Val-Gln-Pro-Lys-Pro-Leu-Glu-Ser-CONH2 (SEQ ID NO:13);

CH3CONH-Met-Ser-Thr-Leu-Gln-Lys-Lys-Gly-Ser-Pro-Asp-Gln-Pro-Leu-Ala-Leu-Pro-Leu-Pro-Glu-Gln-Val-Gln-Glu-Gln-Val-Gln-Glu-Gln-Val-Gln-Pro-Lys-Pro-Leu-Glu-Ser-CONH2 (SEQ ID NO:13);

and derivatives, analogues, homologues, fragments and mixtures thereof.

By "derived from" is meant having an amino acid sequence identical or substantially identical to the sequence of, as used herein, a vascular-associated protein. By "substantially identical to" is meant having an amino acid sequence which differs only by

conservative amino acid substitutions or by non-conservative amino acid substitutions, deletions, or insertions located at positions which do not destroy the biological activity of the peptide.

It is possible to design any number of peptide analogues, having different amino acid sequences, provided that the local charge distribution (and overall net charge) and secondary structure, and hence the biological activity is maintained. Such peptide analogues will generally differ from the native protein sequences by conservative amino acid substitutions (*e.g.*, substitution of Leu for Val, or Arg for Lys, *etc.*) well known to those skilled in the art of biochemistry.

The peptides, once designed, can be synthesized by any of a number of established procedures, including, *e.g.*, the expression of a recombinant DNA encoding that peptide in an appropriate host cell. Alternatively, these peptides can be produced by the established procedure of solid phase peptide synthesis. Briefly, this procedure entails the sequential assembly of the appropriate amino acids into a peptide of a desired sequence while the end of the growing peptide is linked to an insoluble support. Usually, the carboxyl terminus of the peptide is linked to a polymer from which it can be liberated upon treatment with a cleavage reagent. The peptides so synthesized are then labelled with a reagent which enables the monitoring of the peptide after its administration to a patient.

As used herein, the term "substantially corresponds" means a peptide amino acid sequence having approximately 70% homology in amino acid sequence to an apolipoprotein A-IV peptide.

The term "chemical derivative" is meant to include any peptide derived from a peptide of the present invention and in which one or more amino acids have been

chemically derivatized by reaction of one or more functional side groups of the amino acid residues present in the peptide. Thus, a "chemical derivative" as used herein is a peptide which is derived from the peptides identified herein by one or more chemical steps. Examples of derivatized molecules include molecules where free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, thiourethane-type derivatives, trifluroroacetyl groups, chioroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides.

Free hydroxyl groups may be derivatized to form 0-acyl or 0-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For example, 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine.

The term "fragment" refers to any subject peptide having an amino acid sequence shorter than that of any peptide depicted in SEQ ID NO:1-13 and which a fragment retains the appetite suppressant or feeding inhibition properties as the subject peptides. The peptides of the present invention, homologues and analogs thereof may be synthesized by a number of known techniques. For example, the peptides may be prepared using the solid-phase synthetic technique or other peptide synthesis techniques well known to those skilled in the art. The peptides of the present invention might also be prepared by chemical

or enzymatic cleavage from larger portions of the apolipoprotein A-IV molecule or from the entire apo A-IV molecule.

Additionally, the peptides of the present invention may also be prepared by recombinant DNA techniques. For most of the amino acids used to build proteins, more than one coding nucleotide triplet (codon) can code for a particular amino acid residue. This property of the genetic code is known as redundancy. Therefore, a number of different nucleotide sequences may code for a particular subject eating suppressant peptide. The present invention also contemplates a deoxyribonucleic acid (DNA) molecule or segment that defines a gene coding for, *i.e.*, capable of expressing, a subject polypeptide or a subject chimeric polypeptide from which a polypeptide of the present invention may be enzymatically or chemically cleaved.

DNA molecules that encode the subject peptides can be synthesized by chemical techniques, for example, the phosphotriester method of Matteuccie *et al.*, Chem. Soc. 103:3185 (1981). Using a chemical DNA synthesis technique, desired modifications in the peptide sequence can be made by making substitutions for bases which code for the native amino acid sequence. Ribonucleic acid equivalents of the above described DNA molecules may also be used.

A nucleic acid molecule comprising a vector capable of replication and expression of a DNA molecule defining coding sequence for a subject polypeptide or subject chimeric polypeptide is also contemplated.

The peptides of the present invention are preferably chemically synthesized by the Merrifield solid phase technique. In general, the method comprises the sequential addition of one or more amino acid residues to a growing peptide chain. Normally, either the amino

or carboxyl group of the first amino acid residue is protected by a suitable, selectively removable protecting group. A different, selectively removable protecting group is utilized for amino acids containing a reactive side group such as lysine.

Any peptide of the present invention may be used in the form of a pharmaceutically acceptable salt. Suitable acids which are capable of forming salts with the peptides of the present invention include inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, phosphoric acid and the like; and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, fumaric acid, anthranilic acid, cinnamic acid, naphthalene sulfonic acid, sulfanilic acid or the like.

Suitable bases capable of forming salts with the subject peptides include inorganic bases such as sodium hydroxide, ammonium hydroxide, potassium hydroxide and the like; and organic bases such as mono-, di-and tri-alkyl amines (*e.g.*, triethyl amine, diisopropyl amine, methyl amine, dimethyl amine and the like) and optionally substituted ethanolamines (*e.g.* ethanolamine, diethanolamine and the like).

The peptides of the present invention were synthesized using an automatic solid phase peptide synthesizer (Milligen 9050). The synthesis was started by packing a column with a mixture of polystyrene resin (that has attached to it the C terminus amino acid of the target peptide) and glass beads (150-220 micron diameter). The amino acid bound to the resin was protected prior to packing the column and the process of peptide synthesis started by first washing the column with a 20% v/v solution of piperidine / N,N-dimethyl formamide (DMF) in order to deprotect the C terminus residue. The next residue, an FMOC protected L-amino acid (in the form of a pentafluorophenyl ester) was dissolved in

a solution of hydroxybenzotriazole (HOBT/DMF) and delivered to the instrument column. In order to ensure complete coupling, the solution of amino acid/DMF was passed over the column for an extended period of 45-90 minutes. If residue attachment proved difficult due to stearic reasons, the coupling time was extended by a manual modification of the instrument's built in chemical protocols. The protocol for synthesis basically consisted of a number of cycles, each one performing the following operations: deprotection of previous residue with piperidine, washing of the column with DMF, and attachment of the next residue.

In determining the therapeutically effective amount or dose, the effective antioxidant amount or dose of an apolipoprotein (apo) A-IV compound, derivative or fragment thereof, a number of factors are considered by the attending diagnostician, including, but not limited to: the species of the mammal; its size, age, and general health; the response of the individual patient; the particular compound administered; the mode of administration; the bioavailability characteristics of the preparation administered; the dose regimen selected; the use of concomitant medication; and other relevant circumstances.

A therapeutically effective amount will generally vary from about 1 milligram per kilogram of body weight per day (mg/kg/day) to about 5 grams per kilogram of body weight per day (gm/kg/day). A daily dose of from about 1 mg/kg to about 500 mg/kg is preferred.

However, it is understood that the present invention is not limited by any particular theory or proposed mechanism to explain its effectiveness in an end-use application.

In effecting treatment of a patient, an apolipoprotein (apo) A-IV compound, derivative, analog, homolog, fragment and mixtures thereof can be administered in any form or mode

which makes the compound bioavailable in effective amounts, including oral and parenteral routes. For example, the compound can be administered orally, subcutaneously, intramuscularly, intravenously, transdermally, intranasally, rectally, and the like. Oral administration is generally preferred. One skilled in the art of preparing formulations can readily select the proper form and mode of administration depending upon the relevant circumstances.

An apolipoprotein (apo) A-IV compound, derivative, analog, homolog, fragment and mixtures thereof can be administered in the form of pharmaceutical compositions or medicaments which are made by combining an apolipoprotein (apo) A-IV compound, derivative, analog, homolog, fragment and mixtures thereof with pharmaceutically acceptable carriers or excipients, the proportion and nature of which are determined by the chosen route of administration, and standard pharmaceutical practice.

The pharmaceutical compositions or medicaments are prepared in a manner well known in the pharmaceutical art. The carrier or excipient may be a solid, semi-solid, or liquid material which can serve as a vehicle or medium for the active ingredient. Suitable carriers or excipients are well known in the art. The pharmaceutical composition may be adapted for oral or parenteral use and may be administered to the patient in the form of tablets, capsules, suppositories, solution, suspensions, or the like.

The pharmaceutical compositions may be administered orally, for example, with an inert diluent or with an edible carrier. They may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, an apolipoprotein (apo) A-IV compound, derivative or fragment thereof may be incorporated with excipients and used in the form of tablets, troches, capsules, elixirs, suspensions,

syrups, wafers, chewing gums and the like. These preparations should contain at least 4% of an apolipoprotein (apo) A-IV compound, derivative or fragment thereof the active ingredient, but may be varied depending upon the particular form and may conveniently be between 4% to about 70% of the weight of the unit. The amount of the active ingredient present in compositions is such that a unit dosage form suitable for administration will be obtained.

The tablets, pills, capsules, troches and the like may also contain one or more-of the following adjuvants: binders, such as microcrystalline cellulose, gum tragacanth or gelatin; excipients, such as starch or lactose, disintegrating agents such as alginic acid, Primogel, corn starch and the like; lubricants, such as magnesium stearate or Sterotex; glidants, such as colloidal silicon dioxide; and sweetening agents, such as sucrose or saccharin may be added or flavoring agents, such as peppermint, methyl salicylate or orange flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier such as polyethylene glycol or a fatty oil. Other dosage unit forms may contain other various materials which modify the physical form of the dosage unit, for example, as coatings. Thus, tablets or pills may be coated with sugar, shellac, or other enteric coating agents. A syrup may contain, in addition to the active ingredient, sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors. Materials used in preparing these various compositions should be pharmaceutically pure and non-toxic in the amounts used.

For the purpose of parenteral administration, an apolipoprotein (apo) A-IV compound, derivative, analog, homolog, fragment or mixtures thereof may be incorporated into a solution or suspension. These preparations should contain at least

0.1% of a compound of the invention, but may be varied to be between 0.1 and about 50% of the weight thereof. The amount of the active ingredient present in such compositions is such that a suitable dosage will be obtained.

When administered intravenously, the peptide compositions may be combined with other ingredients, such as carriers and/or adjuvants. The peptide can also be covalently attached to a protein carrier, such as albumin, so as to minimize clearing of the peptides. There are no limitations on the nature of the other ingredients, except that they must be pharmaceutically acceptable, efficacious for their intended administration and cannot degrade the activity of the active ingredients of the compositions. The peptide compositions of the invention may also be impregnated into transdermal patches or contained in subcutaneous$_1$ inserts, preferably in a liquid or semi-liquid form A which patch or insert time releases therapeutically effective amounts of one or more of the subject peptides.

The pharmaceutical forms suitable for injection include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the ultimate solution form must be sterile and fluid. Typical carriers include a solvent or dispersion medium containing, for example, water buffered aqueous solutions (*i.e.*, biocompatible buffers), ethanol, polyols such as glycerol, propylene glycol, polyethylene glycol, suitable mixtures thereof, surfactants or vegetable oils. Sterilization can be accomplished by any art-recognized technique, including but not limited to, filtration or addition of antibacterial or antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid or thimerosal. Further, isotonic agents such as sugars or sodium chloride may be incorporated in the subject compositions.

Production of sterile injectable solutions containing the subject peptides is accomplished by incorporating these compounds in the required amount in the appropriate solvent with various ingredients enumerated above, as required, followed by sterilization, preferably filter sterilization. To obtain a sterile powder, the above solutions are vacuum-dried or freeze-dried as necessary.

The solutions or suspensions may also include one or more of the following adjuvants depending on the solubility and other properties of an apolipoprotein (apo) A-IV compound, derivative or fragment thereof: sterile diluents such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylene diaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of toxicity such as sodium chloride or dextrose.

The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

The pharmacological composition will preferably comprise an apolipoprotein (apo) A-IV or derivative or fragment thereof along with a pharmaceutically acceptable carrier, fillers or excipients. The administering step may comprise administering a pharmacological composition comprising an apolipoprotein (apo) A-IV compound, derivative or fragment thereof along with pharmaceutically acceptable carrier, fillers or excipients.

The methods may be by oral administration of the antioxidant composition or a pharmaceutically acceptable salt or derivative thereof into said mammal. The methods

according to the present invention preferable allows the administration of the antioxidant molecule is administered in a unitary dose of from about 1 to about 1000 mg. A unitary dose is generally administered from about 1 to about 3 time a day.

The administering step may comprise parenteral administration of the antioxidant compound or a pharmaceutically acceptable salt or derivative thereof into said mammal. This administration may be by transdermal administration, subcutaneous injection, intravenous injection, intraperitoneal injection, intramuscular injection, intrasternal injection, intrathecal injection, intracerebroventricular injection and infusion techniques.

The method of also comprises administering antioxidant compound or a pharmaceutically acceptable salt or derivative thereof along with a lipophilic compound, such as a lipophilic solvent or carrier. The lipophilic solvent or carrier may be an organic solvent, phosphatidyl choline and cholesterol.

The pharmaceutical compositions of the present invention can be formulated for the oral, sublingual, subcutaneous, intravenous, transdermic or rectal administrations in dosage units and in admixture with pharmaceutical excipients or vehicles. Convenient dosage forms include, among those for oral administration, tablets, powders, granulates, and, among those for parenteral administration, solutions especially for transdermal administration, subcutaneous injection, intravenous injection, intraperitoneal injection, intramuscular injection, intrasternal injection, intrathecal injection and infusion techniques.

The dosage can vary widely as a function of the age, weight and state of health of the patient, the nature and the severity of the ailment, as well as of the administration route. These doses can naturally be adjusted for each patient according to the results observed and the blood analyses previously carried out.

Food Preservative

The apolipoprotein A-IV compounds of the present invention may be made into edible nonionic lipid-soluble additives which are effective antioxidants in food products such as fats, oil, foods, and ingredients of foods without imparting undesirable flavors, aromas, and precipitates.

Oxidation of fats, vegetable oils, carotenoids and their biologically active derivatives, Vitamin A, and of essential oils and other flavorings results in degradation of their quality, and is deleterious to foodstuffs containing the oxidized products.

The art shows many methods of inhibiting lipid oxidation by adding fat-soluble antioxidants to the substrate. The art does not show the stabilization of fats, oils, fatty foods and ingredients of foods employing apolipoprotein A-IV and active deriviatives and fragments in a form effective for such purpose.

A method of preventing oxidation in a lipid-containing food comprising incorporating in the food an oxidation-inhibiting amount of an apolipoprotein A-IV compound, derivative, analog, homolog, fragment and mixtures thereof to protect the food from oxidation.

Generally, the apolipoprotein A-IV molecule is a peptide sequence from about 5 to about 90 amino acids in length.

The food composition may further comprise carriers, fillers, and excipients. Preferably, the apolipoprotein A-IV compound makes up about 0.01% to about 10% of the final weight of the food product. More preferably, the apolipoprotein A-IV compound makes up from about 0.02% to about 5% of the final weight of the food product.

Further, a fat, oil, fatty food or food ingredient substrate stabilized against oxidation with such composition, such a stabilized substrate wherein the substrate includes a carotenoid, and a method of stabilizing a fat, oil, food, or food ingredient substrate which includes the step of introducing into the substrate such a composition as set forth in the foregoing, and such a method wherein the substrate includes a carotenoid.

Pharmaceutical Compositions

The compositions of the present invention may also be used as a method of preventing oxidation in lipid-containing pharmaceuticals. This embodiment involves incorporating in the pharmaceutical an oxidation-inhibiting amount of an apolipoprotein A-IV, derivative, analog, homolog, fragment or mixture thereof. Generally, the apolipoprotein A-IV molecule is a peptide sequence from about 5 to about 90 amino acids in length.

The composition may further include carriers, fillers, and excipients. Preferably, the apolipoprotein A-IV compound makes up about 0.01% to about 25% of the final weight of the pharmaceutical product. More preferably, the apolipoprotein A-IV compound makes up from about 0.05% to about 10% of the final weight of the pharmaceutical product.

Cosmetic or Dermatalogical Preparation

In one embodiment, the present invention provides methods of preventing oxidation in a lipid-containing cosmetic or dermatological composition by incorporating, in a suitable vehicle containing cosmetic or dermatological composition, an oxidation-

inhibiting amount of an apolipoprotein A-IV compound, derivative, analog, homolog, fragment or mixtures thereof.

Generally, the apolipoprotein A-IV molecule is a peptide sequence from about 5 to about 90 amino acids in length. The composition may further comprise carriers, fillers, and excipients. Preferably, the apolipoprotein A-IV compound is present at a concentration between about 0.005% and about 25% by weight with respect to the total weight of the composition. More preferably, the apolipoprotein A-IV compound is present at a concentration between about 0.05% and about 10% by weight with respect to the total weight of the composition.

In another embodiment, the present invention relates to a new antioxidant system based on an apolipoprotein A-IV compound, derivative, analog, homolog, fragment or mixtures thereof for use as an antioxidant system in compositions based on an oleaginous material containing such a system and, principally, cosmetic compositions.

This embodiment generally provides for a cosmetic or dermatological composition containing, in a suitable vehicle, an oxidation-inhibiting amount of an apolipoprotein (apo) A-IV compound, derivative, analog, homolog, fragment or mixtures thereof. Generally, the apolipoprotein (apo) A-IV molecule is a peptide sequence from about 5 to about 90 amino acids in length.

The cosmetic or dermatological composition may be in the form of a suspension or dispersion in a solvent or a fatty substance, or in the form of an emulsion, or in the form of an ointment, a gel, a solid stick or an aerosol foam.

The cosmetic or dermatological composition may additionally contain one or more cosmetic adjuvants such as lower alcohols, polyols, esters of, fatty acids, oils, and waxes,

solvents, silicones, thickeners, emollients, UV-A, UV-B and broad band sunscreens, antifoam agents, hydrating agents, perfumes, stabilizers, surfactants, fillers, sequestrants, anionic, cationic, nonionic and amphoteric polymers and mixtures thereof, propellants, alkalifying and acidifying agents, dyes and metal oxide pigments.

Preferably, the apolipoprotein A-IV compound is present at a concentration between about 0.001% and about 25% by weight with respect to the total weight of the composition. More preferably, the apolipoprotein A-IV compound is present at a concentration between about 0.005% and about 15% by weight with respect to the total weight of the composition.

In another embodiement, the present invention relates to an antioxidant cosmetic system based on apolipoprotein A-IV or at least one of its deriviatives or fragments thereof which contains either at least one basic agent or includes at least one tocopherol or a derivative. Preferably the antioxidant system contains from 0.5 to 20 weight percent of a tocopherol or derivative thereof, 0.5 to 50 weight percent of a basic agent and 0.5 to 90 weight percent of apolipoprotein A-IV and deriviatives and fragments thereof. This antioxidant system is employed in cosmetic or pharmaceutical compositions.

The present invention thus relates to a new antioxidant system based on at least one basic agent characterized by the fact that the system also includes at least one tocopherol or a tocopherol derivative and apolipoprotein A-IV compound, derivative, analog, homolog, fragment or mixtures thereof.

Sodium hydroxide, triethanolamine, basic amino acid may, for example, be used as the basic agent. By basic amino acid is meant a natural basic amino acid such as, for example, lysine, arginine and histidine, their isomeric or racemic forms, as well as synthetic

basic amino acids and derivatives of natural amino acids. Preferably, in accordance with the present invention, lysine or arginine is employed.

By the expression "tocopherol" there is meant not only alpha-tocopherol but also beta , gamma or delta tocopherol as well as their mixtures. Among the tocopherol derivatives mention can be made of the esters of tocopherol such as tocopherol acetate and tocopherol nicotinate.

According to the invention, the antioxidant system is preferably consisting of:

0.5 to 40 percent of a tocopherol or a tocopherol derivative,

0.5 to 50 percent of a basic amino acid and

0.5 to 90 percent of apolipoprotein A-IV and deriviatives and fragments thereof.

The preferred ratio between the concentration of the basic amino acid and the concentration of the tocopherol ranges from 1 to 20.

The compositions according to the invention are provided in the form of oily solutions, water-in-oil or oil-in-water emulsions, optionally anhydrous products, lotions or even microdispersions or ionic or nonionic lipid vesicles. They constitute principally milks for the care of the skin, creams (face creams, hand creams, body creams, sunscreen creams, make-up remover creams, foundation creams), foundation fluids, make-up remover milks, sunscreen milks, bath oils, lipsticks, eyelid make-up, deodorant sticks, *etc.*

For topical application, the pharmaceutical compositions according to the invention comprise vehicles and ingredients necessary to provide, for example, the composition in the form of ointments, creams, milks, pomades and oily solutions.

According to a preferred embodiment, the cosmetic or dermopharmaceutical compositions are provided in a form intended to be topically applied and, in particular, creams intended for the protection of the lipids of the skin against oxidation.

In the compositions according to the invention, the anti-oxidant system, such as defined above, is generally present such that the following proportions, with respect to the total weight of the composition, are established:

Tocopherol or derivative thereof	0.05 to 2%
Basic agent	0.05 to 5%
Apolipoprotein A-IV	0.05 to 8%

The compositions of the invention can also contain active compounds or ingredients conventionally employed in compositions mentioned above, such as surface active agents, dyes, perfumes, astringent products, ultraviolet absorbing products, organic solvents, water, *etc.* These compositions are prepared in accordance with conventional methods.

The compositions of the invention may also contain, in the aqueous phase, various complementary additives such as preserving agents, sequestering agents, gelling agents and the like. The compositions of the invention may also contain, in the lipid phase, various complementary additives such as oils, waxes or gums having, for example, emollient or lubricating properties. The compositions are most often provided in milk, cream or gel form, other modes of presentation not being excluded.

In another embodiment, the present invention provides a process for the preparation of the compositions described above, comprising:

(i) mixing (a) a fatty phase, comprising the lipophilic surfactant, the hydrophilic surfactant, and the fatty acid and (b) an aqueous phase comprising the basic agent and the cosmetically or dermatologically active apolipoprotein A-IV compound by stirring to obtain a mixture; and

(ii) homogenizing the mixture by subjecting the mixture to cavitation.

For cosmetic applications, the compositions of the invention may, moreover, be advantageously used in combination with other compounds displaying retinoid-type activity, with the D vitamins or derivatives thereof, with corticosteroids, with anti-free radical agents, with alpha -hydroxy or alpha-keto acids or derivatives thereof, or alternatively with ion channel blockers, all of these different active agents.

The present invention therefore also features cosmetic compositions comprising a carrier which is cosmetically acceptable and suitable for a topical application and apolipoprotein A-IV. Such cosmetic compositions are advantageously presented in the form of a cream, a milk, a lotion, a gel, microspheres or nanospheres or lipid or polymeric vesicles, a soap or a shampoo.

The concentration of the apolipoprotein (apo) A-IV compound, derivative, analog, homolog, fragment or mixtures thereof in the cosmetic compositions according to the invention advantageously ranges from 0.001% to 30% by weight relative to the total composition.

The medicinal and cosmetic compositions according to the invention may, in addition, contain inert or even pharmacodynamically or cosmetically active additives or combinations of these additives, and, especially: wetting agents; depigmenting agents such as hydroquinone, azelaic acid, caffeic acid or kojic acid; emollients; moisturizing agents

such as glycerol, PEG 400, thiamorpholinone and its derivatives or alternatively urea; antiseborrhoeic or antiacne agents such as S-carboxymethylcysteine, S-benzylcysteamine, salts or derivatives thereof, benzoyl peroxide; antibiotics such as erythromycin and esters thereof, neomycin, clindamycin and esters thereof, tetracyclines; antifungal agents such as ketoconazole or 4,5-polymethylene-3-isothiazolidones; agents promoting hair regrowth, such as Minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide) and derivatives thereof, Diazoxide (7-chloro-3-methyl-1,2,4-benzothiadiazine-1,1-dioxide) and Phenytoin (5,4-diphenyl-2,4-imidazolidinedione); non-steroidal anti-inflammatory agents; carotenoids and especially beta -carotene; anti-psoriatic agents such as anthralin and derivatives thereof; and, lastly, 5,8,11,14-eicosatetraynoic and 5,8,11-eicosatrynoic acids and esters and amides thereof.

The compositions according to the invention may also contain taste- or flavor-enhancing agents, preservatives such as parahydroxybenzoic acid esters, stabilizing agents, moisture regulating agents, pH regulating agents, osmotic pressure modifying agents, emulsifying agents, UV-A and UV-B screening agents, antioxidants such as alpha - tocopherol, butylated hydroxyanisole or butylated hydroxytoluene.

The following examples illustrate and explain the present invention but should not be taken as limiting the present invention in any regard.

Examples.

Over-expression of apolipoprotein (apo) A-IV in transgenic mice confers significant protection against atherosclerosis, despite a more severe atherogenic lipid profile (Duverger, N., Tremp, G., Caillaud, J. M., *et al.* Protection against atherogenesis in mice mediated by human apolipoprotein AIV. *Science* **273**, 966-968 (1996); Cohen, R.

D., Castellani, L. W., Qiao, J. H., Lenten, B. J. V., Lusis, A. J. & Reue, K. Reduced aortic lesions and elevated high density lipoprotein levels in transgenic mice overexpressing mouse apolipoprotein A-IV. *J. Clin. Invest.* **99**, 1906-1916 (1997)).

Fasting lymph was used to mimic conditions in the interstitial fluid, a potential site for lipoprotein oxidation *in vivo*. ApoA-IV greatly inhibited copper induced oxidation of lymph. Using purified LDL as the substrate, the addition of apoA-IV prevented the copper-mediated formation of conjugated dienes. Rat and human apoE or bovine serum albumin offered little protection. ApoA-IV was also effective during the propagation phase of LDL oxidation. The methods of the present invention utilize apoA-IV as a potent endogenous antioxidant.

Over-expression of either human or mouse apoA-IV (a plasma protein) in transgenic mice confers significant protection against diet-induced atherosclerosis in cholesterol-fed animals (Duverger, N., Tremp, G., Caillaud, J. M., *et al.* Protection against atherogenesis in mice mediated by human apolipoprotein AIV. *Science* **273**, 966-968 (1996); Cohen, R. D., Castellani, L. W., Qiao, J. H., Lenten, B. J. V., Lusis, A. J. & Reue, K. Reduced aortic lesions and elevated high density lipoprotein levels in transgenic mice overexpressing mouse apolipoprotein A-IV. *J. Clin. Invest.* **99**, 1906-1916 (1997)) and also in apoE-deficient mice despite a more severe atherosclerotic lipid profile (Duverger). A hallmark of atherosclerosis is the presence of oxidized lipids in lipoproteins and in the lesions (Chisolm III, G. M. & Penn, M. S. in *Atherosclerosis and coronary artery disease* (eds Fuster, V., Ross, R. & Topol, E.J.) Vol. 1, 129-149 (Lippincott-Raven, Philadelphia, 1996); Berliner, J. A. & Heinecke, J. W. The role of oxidized lipoproteins in

atherogenesis. *Free Radic. Biol. Med.* **20**, 707-727 (1996)). In this study, we assessed the role of apoA-IV in protecting against lipid oxidation.

Initial experiments used fasting intestinal lymph as the substrate to mimic conditions in the interstitial fluid, a potential site of lipoprotein oxidation. Maximal lipid peroxidation (the generation of TBARS) was achieved by incubating the lymph with buffer containing 100 μM Cu^{2+} at 37°C for 3 h. Under these experimental conditions, addition of apoA-IV inhibited the oxidation of lymph lipids in a dose-dependent manner (Fig. 1). The anti-oxidative effect was apoA-IV specific as neither BSA nor rat apoE offered any protective effect at 20 μg/ml concentration (data not shown). The inability of apoE to protect against Cu^{2+} induced lipid oxidation observed herein is contradictory to previous observation that apoE prevents lipoprotein oxidation (Miyata, M. & Smith, J. D. Apolipoprotein E allele-specific antioxidant activity and effects on cytotoxicity by oxidative insults and beta-amyloid peptides. *Nature Genetics* **14**, 55-61 (1997)). The discrepancy may be explained by differences in experimental conditions of the two studies.

One explanation for our observation may be the ability of apoA-IV to complex with copper ions, thus preventing divalent cation-induced lipid oxidation events. This is unlikely since excess Cu^{2+}, achieved by extensive dialysis of apoA-IV against 100 μM Cu^{2+}, failed to overcome the anti-oxidative effects of apoA-IV (data not shown). ApoA-IV's protection against lipid oxidation is physiologically relevant because maximal protection against lipid oxidation was achieved with an apoA-IV concentration that is significantly lower than its lymph (1/10th plasma) and plasma concentration.

Apo A-IV was also effective in inhibiting LDL oxidation in a dose-dependent manner (Fig. 1). The fact that apoA-IV inhibits LDL oxidation at 5 μg/ml underscores its potential importance in protecting against atherogenesis by preventing LDL oxidation.

The ability of apoA-IV to prevent LDL oxidation was further compared with that of apoE by monitoring the formation of conjugated dienes. As shown in Fig. 2, the addition of 2.5 μg/ml of apoA-IV increased the lag time of Cu^{2+}-induced conjugated diene formation in LDL by 3-fold and 5 μg/ml of apoA-IV totally abolished conjugated diene formation. In contrast, rat and human apoE (20 μg/ml) increased the lag time of Cu^{2+}-induced conjugated diene formation in LDL by only 1.3- and 1.5-fold, respectively (Fig. 2).

We also determined if apoA-IV can inhibit LDL oxidation during the propagation phase of the oxidative process. As shown in Fig. 3, addition of apoA-IV during the propagation phase resulted in a dose-dependent inhibition of LDL oxidation. A linear inverse relationship ($R^2 = 0.9955$) was observed between the rate of conjugated diene formation and the amount of apoA-IV present (Fig. 3 inset), suggesting a direct effect of apoA-IV in interfering with the oxidation process.

In conclusion, apoA-IV may play an important role in preventing the oxidation of lipoproteins *in vivo*. Although many antioxidants exist in nature, apoA-IV is unique because: (i) unlike exogenous anti-oxidants such as a-tocopherol, ascorbate and b-carotene, apoA-IV is synthesized in the body and thus is a natural antioxidant; (ii) apoA-IV's production by the intestine is directly correlated with fat intake; and (iii) apoA-IV is amphipathic and is distributed in lipoprotein-bound and lipoprotein-free forms in circulation. In view of observations that apoA-IV is a more potent anti-oxidant than

apoE, a well established anti-atherogenic protein, the results suggest that therapeutic treatment aimed at increasing apoA-IV levels may be a fruitful strategy for the reduction of coronary heart disease.

Methods

Purification of lipoproteins and apolipoproteins. Human LDL (d = 1.019 - 1.063 g/ml) Mwas isolated from the plasma of normal blood donors by density gradient ultracentrifugation and stored in saline-EDTA. Before use, EDTA was eliminated by dialysis against PBS. LDL concentration is expressed as mg protein as determined by the Lowry assay. Rat apolipoproteins were purified from rat plasma as previously described[8].

Collection of lymph fluid. Intestinal lymph duct of adult male Sprague-Dawley (SD) rats was cannulated. After surgery, rats were infused intraduodenally with a glucose-saline solution and intestinal lymph was collected on ice, and stored at -20° C.

Assay of lipid oxidation. Lipid oxidation of lymph and LDL were assayed by the formation of thiobarbituric acid reactive substances (TBARS) (Hulea, S. A., Waswicz, E. & Kummerow, F. A. Inhibition of metal-catalyzed oxidation of low-density lipoprotein by free and albumin-bound bilirubin. *Biochim. Biophys. Acta* **1259**, 29-38 (1995)). Fasting lymph (diluted 20 times) or LDL (50 µg/ml) was oxidized, with or without purified apolipoproteins, in the presence of 100 or 10 µM $CuSO_4$ for 3 h at 37°C. In addition, LDL oxidation was also determined by the formation of conjugated dienes (Kleinveld, H. A., Hak-Lemmers, H. L. M., Stalenhoef, A. F. H. & Demacker, P. N. M. Improved measurement of low-density-lipoprotein susceptibility to copper-induced oxidation: